Indian Journal of Biochemistry & Biophysics
Vol. 57, April 2020, pp. 178-184

Effect of Indian honey on expression of p53 and cyclin B1 in HeLa cells

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Received 11 October 2019; *revised 08 November 2019*

Honey is a natural product collected from the nectar by honey bees and has a wide array of therapeutic important compounds called as phytochemicals. Cancer is one of the leading causes of death worldwide. Existing conventional cancer therapy which is mainly comprised of chemotherapeutic agents has side effects. Honey due to the presence of phytochemicals especially flavonoids and phenolic acids like apigenin, chrysin, quercetin and caffeic acid are found to inhibit the cancer growth. This effect varies among different honeys since they are dependent on the phytochemical composition and their concentrations in honey, which varies with respect to floral origin from which honey has been collected, geographical and climatic conditions. Thus, in the current study, phytochemical characterization of Jambhul, Rubber, Litchi and Drumstick honey samples along with their effect on the expression of proteins playing a vital role in the event of cell cycle and apoptosis *i.e.* Cyclin B1 and p53 proteins were determined by Flow cytometry. It was found that these honey samples contain apigenin, chrysin, quercetin and caffeic acid. These honeys exhibited an increase in expression of p53 whereas, expression of Cyclin B1 protein was reduced in HeLa cells treated with honey samples. The presence of phytochemical markers exhibiting promising anticancer potential along with the ability of alterations in the expression of these proteins proves that these honey samples have a potent anticancer activity which enhances the chances of these Indian honeys to be utilized in the treatment of cervical cancer.

Keywords: Apigenin, Apoptosis, Caffeic acid, Chrysin, Cyclin B1, Oncogenes, p53, Quercetin

Honey has been used for many centuries not only as a sweetener but also as a food preservative and therapeutic product[1]. Honey has been a common sweetener and a powerful medicinal tool for centuries[2,3]. In nature, honey is found to be of different types. In monofloral honey, also called as a unifloral, honey bees forage predominantly on one type of plant and thus it is named according to the plant *e.g.* sunflower honey. India has a wide array of biodiversity with respect to flora and fauna. Hence, in India various apiaries have a collection of honey from various floral sources with wide variations in physicochemical, functional and rheological characteristics[4]. Honey is comprised of over 200 compounds[5]. Honey is a complex natural sweetener composed mainly of carbohydrates (60–85%) and water (12–23%). In addition to that, it also contains small amounts of other compounds, such as organic acids, minerals, vitamins, enzymes, proteins, amino acids, volatile compounds and several bioactive substances *i.e.* phenols and flavonoids[6,7]. Each of these minor constituents is known to have distinctive nutritional or medicinal properties and the unique blend of these accounts for the varied as well as different applications of natural honeys[8]. Phytochemicals are chemicals produced by plants through primary or secondary metabolism[9]. Phytochemicals are also regarded as research compounds rather than essential nutrients since they possess wide variety of medicinal properties[10]. Though, polyphenols are divided into several classes, according to the phenolic structural features, in honey they are mainly flavonoids, phenolic acids and phenolic acid derivatives[11,12]. Honey contains a wide range of phytochemicals including polyphenols which

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Abbreviations: %, Percentage; °C, Centigrade; CO_2, Carbon dioxide; DMEM, Dulbecco's Modified Eagle's Medium; FACS, Fluorescence activated cell sorting; h, Hours; HPTLC, High-Performance Thin-Layer Chromatography; IC_{50}, Inhibitory concentration 50; mg, Milligrams; Min, Minute; ml, Millilitre; p53, Tumor protein p53; PBS, Phosphate buffer saline; µg, Microgram

act asantioxidants[13]. The existence and concentrations of these polyphenols in honey can vary depending mainly on the floral source from which it has been collected, the climatic and geographicalconditions[14].

With rapid population growth worldwide, the rising prominence of cancer as a leading cause of death partly reflects marked declines in mortality rates of stroke and coronary heart disease relative to cancer, in many countries[15]. Cervical cancer is the second most common cancer among women[16]. Due to the unregulated cellular proliferation induced by the degradation of p53 and pRb, the HPV infected cells evolve towards a premalignant condition called dysplasia[17]. Several studies have demonstrated that honey can be a potential agent against oxidative stress disorders including cardiovascular disease, cancer, diabetes, hepatic and renalfailure[18]. The mechanism on how honey exhibits anticancer effect is an area of great interest in recent years[19]. The anticancer activity is due to the phytochemical composition of honey and hence it varies among honeys which depends on the factors such as climatic conditions, source *etc*. Thus in this study, we have performed the phytochemical characterization of the four Indian unifloral honey samples along with the estimation of expression of proteins *i.e.* p53 and Cyclin B1 playing vital role in cell cycle and apoptosis in order to find out the effect of honey at the molecular level in HeLa cells treated with these four unifloral honey samples.

Materials and Methods

Honey samples

Four different types of unifloral Indian honey *i.e.* Jambhul, Rubber, Litchi and Drumstick honey were procured from standard apiaries of India. They were stored at room temperature in dry and sterile conditions.

Extraction of honey samples

Liquid – liquid extraction of these four honey samples was carried out by using organic solvents of different polarities *i.e.* Ethyl acetate, Diethyl ether, and Chloroform. These extracts were then loaded on High-Performance Thin-Layer Chromatography (HPTLC) system for the quantitative estimation of the phytochemicals[20,21].

Quantitative analysis of phytochemicals

Phytochemical components of these four honey samples were quantified by High- Performance Thin-Layer Chromatography (HPTLC). It was performed at Anchrom Test Labs Private Limited, Mumbai, India. Coated uniform silica gel 60 F254 TLC plates of thickness 0.2 mm and size 20 × 10 cm were used on which honey extracts were loaded along with standards from sigma *i.e.* quercetin, chrysin, apigenin and caffeic acid by Linomat 5 semi-automatic sampler. The solvent system used for chrysin, apigenin and caffeic acid are Toluene: Ethyl acetate: Formic acid (6:4: 0.3) and for better resolution of quercetin it was Toluene: Methanol: Ethyl acetate: Formic acid (5:1:4: 0.3). Visual imprints of the TLC plates were taken using Camag TLC visualizer. Densitometric evaluation of the plate was carried out in a Camag TLC scanner using the WINCATS software system. The concentration of each phytochemical in samples was quantified by comparing the area under curve (AUC) values of samples with the area under curve (AUC) values of respective standards[22].

Cell culture

Human cervical cancer cells *i.e.* HeLa cell line was maintained as monolayer cultures in DMEM cell culture media supplemented with 10% fetal bovine serum, 1X Antibiotic-Antimycotic solution. The cell cultures and experimental plates were maintained at 37°C in 5% CO_2.

Protein expression study by flow cytometry

This assay was performed using PE Mouse Anti-Human Cyclin B1 and Alexa Fluor 488 Mouse Anti-p53 antibodies (BD pharmingen). Cells treated with IC_{50} concentrations*i.e.*2.4 ± 0.3%, 2.9 ± 0.2%, 4.0 ± 0.8%, 4.2 ± 0.6% of Jambhul, Rubber, Litchi and Drumstick honey, respectively, for 48 h. were trypsinized and were fixed with chilled 1% paraformaldehyde for 15 min at 4°C. Cells were washed twice with FACS I buffer containing 1X PBS with sodium azide and fetal bovine serum. Control cells were processed exactly as honey treated cells. 1 × 10^6 cells were suspended in 100 µL FACS buffer II containing saponin, 1X PBS with sodium azide and fetal bovine serum for 15 min. 20 µL of each of PE Mouse Anti-Human Cyclin B1 and Alexa Fluor 488 Mouse Anti-p53 antibodies was added to cells as per the instructions given in the product sheet. After incubation of 45 min in dark, cells were washed twice with FACS buffer I and were centrifuged at 1000 rpm for 10 min. Cells were then resuspended in 0.5 ml of chilled FACS buffer I and were acquired on Flow

Cytometer. The analysis was done by using CXP 500 software provided with the Backmen Flow Cytometer. The assay was performed in triplicates[23].

Statistical analysis

The values of the data obtained from three independent experiments are expressed as mean ± standard deviation. Statistical evaluation was performed using the ANOVA test and P <0.05 was considered significant.

Results

Quantitative analysis of phytochemicals

Quantitative estimation of phytochemical markers *i.e.* quercetin, chrysin, apigenin and caffeic acid in honey samples was done by using respective standards on High- Performance Thin-Layer Chromatography (HPTLC). To quantify them, densitometric evaluation of the plate was carried out in a Camag TLC scanner using the WINCATS software system. The concentration of phytochemicals in samples was calculated by Area under the curve of samples and that of corresponding standards of respective phytochemicals. Among these four Indian unifloral honey samples, quercetin was detected only in chloroform extract of Jambhul honey at the concentration of 0.3 µg/mg of honey. Caffeic acid was identified and quantified in all four honey samples. Among the four honey samples of this study, caffeic Acid was predominantly detected in Rubber honey in the range of 40-67 µg/mg of honey. Similarly, apigenin was identified and quantified in all four honey samples. This was also predominantly detected in Rubber honey in the range of 3-8 µg/mg of honey among the four honey samples. Chrysin was identified predominantly in Jambhul honey among four honey samples in the concentration range of 1-2 µg/mg of honey (Fig. 1). From the data obtained it was observed that Jambhul honey showed the presence of all four phytochemicals *i.e.* quercetin, chrysin, apigenin and caffeic acid. In addition to this it was also found that, out of these four phytochemicals, quercetin and chrysin were predominantly detected in Jambhul honey as compared to other honey samples of this study.

Protein expression study by flow cytometry

Protein expression studies were performed by flow

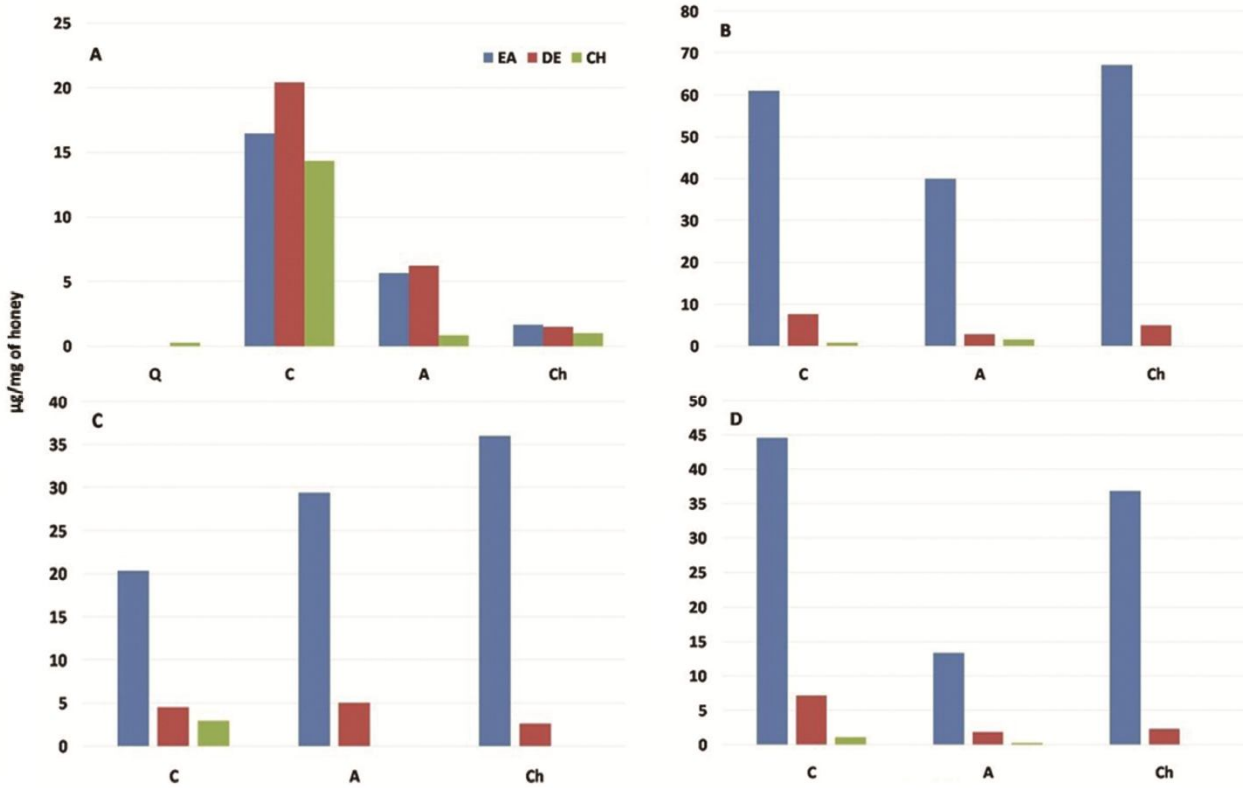

Fig. 1 — Quantitative estimation of phytochemicals in extracts of honey samples by using HPTLC. A: Jambhul honey, B: Rubber honey, C: Litchi honey, D: Drumstick honey; EA: Ethylacetate, DE: Diethyl ether, CH: Chloroform; Q: Quercetin, Ch: Chrysin, A: Apigenin,C: Caffeic acid

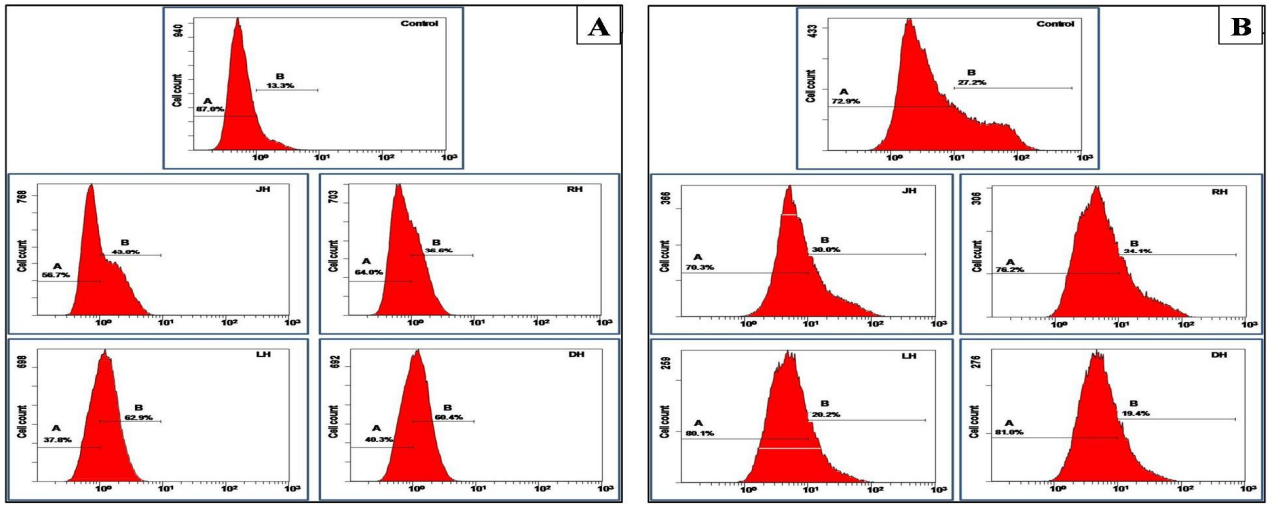

Fig. 2 — Effect of honey on the expression of proteins. The expression of p53 protein (A) and Cyclin B1 protein (B) was determined in HeLa cells treated with honey samples for 48 h by Flow cytometry.Control: Untreated cells; JH: Cells treated with Jambhul honey; RH: Cells treated with Rubber honey; LH: Cells treated with Litchi honey; DH: Cells treated with Drumstick honey.

cytometry. After 48 h.of honey treatment HeLa cells, both treated and untreated cells were stained with antibodies tagged with fluorochrome and were acquired on a flow cytometer for the analysis of expression of proteins playing important role in events of the cell cycle and apoptosis. Cyclin B1 protein and p53 protein expression in cells treated with all four Indian unifloral honey samples were analyzed by using CXP 500 software of flow cytometer. All four honey samples induced increase in the p53 protein expression in Hela cells (Fig. 2A). Expression of Cyclin B1 protein was decreased post-treatment in HeLa cells in Rubber, Litchi and Drumstick honey (Fig. 2B).

On treatment with Jambhul, Rubber, Litchi and Drumstick honey expression of p53 protein in HeLa cells was enhanced significantly which was indicated by the positive cell population of 43.3±0.8% (P <0.0001), 36.0±0.8% (P <0.0001), 62.4±0.7% (P <0.0001), 60.0±0.6% (P <0.0001), respectively whereas, cell population (%) of untreated HeLa cells was 12.5±1.1%. Treatment of Rubber, Litchi and Drumstick honey induced significant reduction in the expression of Cyclin B1 protein in HeLa cells indicated by the positive cell population of 23.8±0.4% (P <0.05), 20.5±0.4% (P <0.01), 20.7±1.8% (P <0.01), respectively, whereas, cell population (%) of untreated HeLa cells was 28.1±1.2%.The positive cell population of 30.9±1.2% was seen in cells treated with Jambhul honey.

Discussion

In spite of considerable research inputs, cancer is still a challenge to prevent and treat[24]. There is a huge demand for cancer treatment and various options available for cancer therapy. Conventional methods to treat cancer have severe side effects. Most of the drugs that are currently used as chemotherapeutic agents are not highly effective and may lose their efficacy due to the development of drug resistance[25,26]. Hence, therapies that can prevent progression to malignancy, reduce the required dosage of conventional drugs or lessen the severity of adverse effects are of considerable benefit. Approximately 90%–95% of cancer cases are thought to be related to the environment and lifestyle of an individual. This highlights the potential role of diet in carcinogenesis. In recent years, there has been increase in interest in the search for chemo preventive and chemo therapeutic agents derived from food or natural products. The relative safety of the food derived compounds makes it an attractive alternative to conventional cancer therapy[24]. One such commonly used foodstuff since ancient times is honey[27].

Almost all natural kinds of honey contain flavonoids such as apigenin, chrysin, quercetin and phenolic acids such as caffeic acid[28]. In order to understand the phytochemical composition of honey on which its medicinal properties are based, we quantified four phytochemicals of four different

unifloral Indian honey samples. In our study, we have performed the High Performance Thin Layer Chromatography (HPTLC) in order to estimate the phytochemical components *i.e.* quercetin, chrysin, apigenin and caffeic acid of four Indian unifloral honey samples quantitatively. We selected these four biologically important phytochemicals which have potent anticancer activity. Quantification of each one of these phytochemicals from all the samples was done by densitometric evaluation of the TLC plate using a Camag TLC scanner using the WINCATS software system. Among the four unifloral Indian honey samples; quercetin was detected only in Jambhul honey at the concentration of 0.3 µg/mg of honey. Caffeic Acid was identified and quantified in all four honey samples. It was predominantly detected in Rubber honey (40-67 µg/mg of honey) among the four honey samples of this study. Similarly, apigenin was identified and quantified in all four honey samples. This was also predominantly detected in Rubber honey (3-8 µg/mg of honey) among the four honey samples. Chrysin was identified predominantly in Jambhul honey among four honey samples in the concentration range of 1-2 µg/mg of honey (Fig. 1). From the data obtained it was observed that, Jambhul honey showed the presence of all four phytochemicals *i.e.* quercetin, chrysin, apigenin and caffeic acid. In addition to this it was also found that, out of these four phytochemicals, quercetin and chrysin were predominantly detected in Jambhul honey as compared to other honey samples of this study. Jaganathan *et al.* (2010) screened four Indian honey samples for their phenolic acids in which they reported caffeic acid was in the range of 0.11 – 0.37µg/g of honey[29]. Angira Das *et al.* (2015) screened seven (sesame) honey samples from West Bengal, India for detection of flavonoid content in which they identified quercetin and apigenin[30]. Rosemary honey and citrus honey samples were screened for the presence of flavonoids by Tom & Barbed *et al.* (1993). They identified quercetin, chrysin, apigenin by using respective standards[31]. From the published data it is clear that the phytochemical components and their concentrations vary among different types of honey. Similar to other published reports, all four honey samples of this study, showed the presence of phytoconstituents. Concentrations detected in these four honey samples showed similar variations among them to that of available literature. These possible differences in the concentration of these phytochemicals within four honey samples and also with the compared results of other studies from literature are due to geographical and climatic differences. Since these phytochemicals have been transported to honey from the nectar, these variations as have been already reported to be related to their floral sources in addition to geographical and climatic conditions[14, 32,33].Presence of phytochemicals *i.e.* quercetin, chrysin, apigenin and caffeic acid which are proven anticancer agents in honey samples of this study enhances the chances of use of these honeys to serve as a good source of these natural antioxidants present in them which may be helpful and can be explored in reducing the incidences of cervical cancer.

In addition, to inhibiting cell proliferation, honey has also been reported to alter cell cycle progression and induce apoptosis in cancer cell lines[25,34]. In recent years, an increasing number of studies have been reported that the expression of Cyclin B1 may be correlated with the poor outcome of patients with various cancers, including breast carcinoma, prostatic cancer, pancreatic malignancy, lung carcinoma, laryngeal cancer as well as hepatocellular cancer, gastric cancer, colorectal cancer and esophageal cancer[35]. Oncogenes and tumor suppressor genes play a crucial role in regulation of the cell cycle, particularly those of the p53 pathways which are involved in the restriction checkpoint[36]. Usually, the cellular level of p53 is low but DNA damage can lead to rapid induction of p53 activity. p53 also mediates the dissociation of CDK1-cyclin B1 complexes by induction of Gadd45 (growth arrest and DNA damage inducible gene)[37]. Evidence to date shows a promising effect of honey and its constituents in promoting p53 expression[24]. In this study, in order to understand the mechanism of action of honey on HeLa cell cycle arrest and apoptosis we performed Cyclin B1 & p53 expression experiment by flow cytometry. Flurochrome labelled antibody was used for detection of these proteins by flow cytometry. Expression of these proteins in cells treated with IC_{50} concentrations of honey samples for 48 h. was compared with those in untreated cells. We found increase in the expression of p53 protein. p53 is a tumor suppressor and an important transcription factor regularly inactivated in many forms of human tumors. It mediates tumor

suppression by modulating the transcription of many genes involved in the regulation of apoptosis[33]. Outcomes of our study supports and confirms the data obtained in our previous experiment of cell cycle analysis in which significant increase in the cell population in Sub G0G1 phase indicated the induction of apoptosis due to the treatment of honey in cervical cancer cells[38]. In current study, the expression of Cyclin B1 protein was reduced by the honey treatment. Post honey treatment, reduction in Cyclin B1 indicates that the treatment of honey intervened the process of cell cycle in treated cells which is already has been observed in our previous experiment of cell cycle analysis where the honey has induced a cell cycle arrest at G2M phase in HeLa cells[38]. *In vitro* study on colon carcinoma cell lines showed that a pure unfractionated Indian honey induced apoptosis. The authors analysed the expression of proteins involved in apoptosis, concluding that the protein expression was affected in a time dependent manner with the greatest difference being observed at 48 h with an increase in p53[39]. Induction of p53 in cancer cells was reported in colon, breast and liver cancer cells treated with honey[40]. Chiang *et. al.* did flow cytometric analysis which indicated apoptosis. Further they observed increasing accumulation of p53 in the treated cancer cells[41]. It has been demonstrated that Cyclin B1 is required for survival and proliferation of breast and cervical cancer cells and thus down regulation of Cyclin B1 inhibits proliferation of tumor cells *in vitro* as well as *in vivo*[42]. A similar effect has been observed in the current study in HeLa cells. The alteration in expression of p53 protein was also accompanied with the decrease in the expression of Cyclin B1 protein in cervical cancer cells after the honey treatment of 48 h. Findings of this study are consistent with the results on the inhibition of cancer cell proliferation, G2M cell cycle arrest and down regulation of Cyclin B1 expression along with the increase in expression of p53 protein, all of which suggest that these Indian unifloral honey samples has the potential to inhibit the growth of HeLa cells by modulating the expression of Cyclin B1 and p53 protein. Hence, these findings emphasizes the anticancer effect of Indian honey on cervical cancer cells. Further studies are required to estimate the anticancer effect of these honey samples *in vivo*.

Conclusion

From this study we conclude that all four Indian honey samples possess potent anticancer activity against cervical cancer cells by modulating the expression of Cyclin B1 and p53 protein. The presence of phytochemicals in honey samples might have a role in anticancer activity.

Conflict of Interest

All authors declare no conflict of interest.

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